Volume 312, number 2,3, 245–248 FEBS 11737 November 1992

Primary structure required for the inhibition of smooth muscle myosin light chain kinase

Mitsuo Ikebe[a], Sheila Reardon[a] and Fredric S. Fay[b]

[a]*Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44106-4970, USA* and [b]*Department of Physiology, University of Massachusetts, Worcester, MA 01605, USA*

Received 26 August 1992; revised version received 21 September 1992

Myosin light chain kinase (MLCK) contains the autoinhibitor sequence right next to the N-terminus side of the calmodulin binding region. In this paper, the structural requirement of the inhibition of MLCK activity was studied using synthetic peptide analogs. Peptides Ala-783–Lys-799 and Ala-783–Arg-798 inhibited calmodulin independent MLCK at the same potency as the peptide Ala-783–Gly-804. Deletion of Arg-797–Lys-799 or substitution of these residues to Ala markedly increased the K_i while the substitution of Lys-792 and Lys-793 to Ala and the deletion of Lys-784–Lys-785 did not affect the inhibitory activity of the peptides. The results suggest that Arg-797–Arg-798 are especially important for the inhibitory activity among other basic residues in the autoinhibitory region.

Myosin light chain kinase; Calmodulin; Protein kinase; Phosphorylation; Synthetic peptide

1. INTRODUCTION

Activation of contractile machinery of smooth muscle is triggered by phosphorylation of the regulatory light chain of myosin [1,2]. Phosphorylation of the regulatory light chain of myosin is catalyzed by a Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK). The structure–function relationships of smooth muscle MLCK are greatly assisted by the determination of the complete amino acid sequence [3–5]. In the central portion of the molecule, the sequences commonly observed in various protein kinases [3–5] are found and, therefore, it is assigned that the catalytic domain exists at the central part of the molecule. Calmodulin binding peptide was isolated by Lukas et al. [6] and the sequence correlates with residues 797–816 of chicken gizzard MLCK sequence [3,4] which lies towards the C-terminal side of the catalytic domain. Kemp et al. [7] found that residues 788–811 showed significant homology to the N-terminal sequence of the regulatory light chain of myosin and that a synthetic peptide analog of this region inhibited Ca^{2+}/calmodulin-dependent MLCK activity. Based upon this result, Kemp et al. [7] proposed that this region serves as a pseudo-substrate inhibitor. We demonstrated [8] that the proteolysis of the kinase yielded a 64 kDa inactive fragment which was converted to a 61 kDa active Ca^{2+}/calmodulin-independent kinase by further proteolysis. The Ca^{2+}/calmodulin-independent MLCK in which the regulatory site is removed was strongly inhibited by the pseudo-substrate inhibitor peptide residues 783–804 [8]. These results show that there is an autoinhibitory domain at the N-terminal side of the calmodulin binding region. However, it is controversial whether or not the autoinhibitory domain functions as a pseudo-substrate inhibitor. Recent studies on skeletal muscle and non-muscle myosin light chain kinase suggested [9,10] that the inhibitory region may not simply function as a pseudo-substrate inhibitor.

In this paper, the structure required for the autoinhibition of smooth muscle MLCK was studied using various synthetic peptide analogs of the autoinhibitory domain of MLCK. The obtained information of the structural requirement of the peptide inhibitor is directly relevant to in vivo application to study the physiological function of the kinase [11] and this is another objective of this paper.

Correspondence address: M. Ikebe, Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44106-4970, USA.

2. MATERIALS AND METHODS

2.1. *Proteins and peptides*

MLCK [8] and calmodulin [12] were prepared from turkey gizzard and bull testes, respectively. Constitutively active MLCK was obtained by tryptic digestion of MLCK as described previously [8]. Smooth muscle CaMPKII was prepared from chicken gizzard as described previously [13]. Smooth muscle S-1 was prepared from turkey gizzard myosin [14] by *S. aureus* protease digestion as described previously [15]. Caldesmon was prepared from turkey gizzard according to the method of Bretcher [16].

2.2. *Others*

Phosphorylation of proteins was assayed as described by Walsh et al. [12]. Protein concentration was estimated by biuret or spectrometric measurements for calmodulin E_{277nm} = 1.9; MLCK, E_{280nm} = 11.4.

3. RESULTS AND DISCUSSION

It has been found [17] that the amino acid sequence at the N-terminal side of the regulatory site of MLCK is homologous to the sequence at the N-terminal side of the phosphorylation site of the regulatory light chain of myosin in terms of their abundance of basic amino acid residues. Based upon this finding, it was proposed [7,17] that this basic amino acid rich region may act as a pseudo-substrate inhibitor. We synthesized various synthetic peptide analogs of the inhibitory region of smooth muscle MLCK (Table I) and studied the requirement of basic amino acid residues for the inhibitory activity. The basic amino acid residues of the inhibitory region analog peptide were either deleted or substituted for alanine (Table I). Fig. 1 shows the inhibition of the Ca^{2+}/calmodulin-independent constitutively active MLCK activity by synthetic peptide analogs. The peptides Leu-786–Lys-802 and Lys-788–Lys-799 inhibited the constitutively active MLCK with a similar potency to the peptide Ala-783–Gly-804. This suggests that a cluster of the basic amino acid residues Lys-784 and Lys-785 are not essential for the inhibitory activity of the peptides. The results also suggest that the C-terminal side of the cluster of basic amino acid residues, Trp-800–Gly-804, are not important for the inhibitory activity. Substitution of Lys-792 and Lys-793 did not significantly alter the inhibitory activity of the peptides suggesting that these basic amino acid residues are not important for the inhibitory activity of the peptides. On the other hand, both deletion of a cluster of the basic amino acid residues, Arg-797–Lys-799, and substitution of Arg-797–Lys-799 for alanine significantly decreased the inhibitory activity of the peptides. To further analyze the importance of these residues, a single amino acid residue of Lys-799 was deleted and the effects of the deletion on the inhibitory activity were examined. However, the deletion of Lys-799 did not decrease the inhibitory activity of the peptide. These results suggest that Arg-797 and Arg-798 are important for the inhibitory activity of the peptide, while other basic amino acid residues in the inhibitory region of MLCK are not essential for the inhibitory activity. It was shown [18] that the peptide Asp-777–Lys-793 has little inhibitory activity against the constitutively active MLCK with a K_i of 140 μM. Therefore, although the significance of Lys-788 and Arg-800 on the inhibitory activity was not directly determined, it is unlikely that these residues significantly contribute to the inhibitory activity of the peptides. It should be noted, however, it was shown [8] that the peptide Ala-796–Ser-815 which contains a cluster of basic amino acid residues Arg-797–Lys-799 is not a strong inhibitor for Ca^{2+}/calmodulin-independent MLCK suggesting that the amino acid residues Arg-797–Arg-798 are important but not sufficient to express the inhibitory activity. The N-terminal side structure of Arg-797–Arg-798 is also required for the inhibitory activity. The importance of the basic amino acid residues in the autoinhibitory region for the inhibitory activity was originally pointed out by Kemp and co-workers [7].

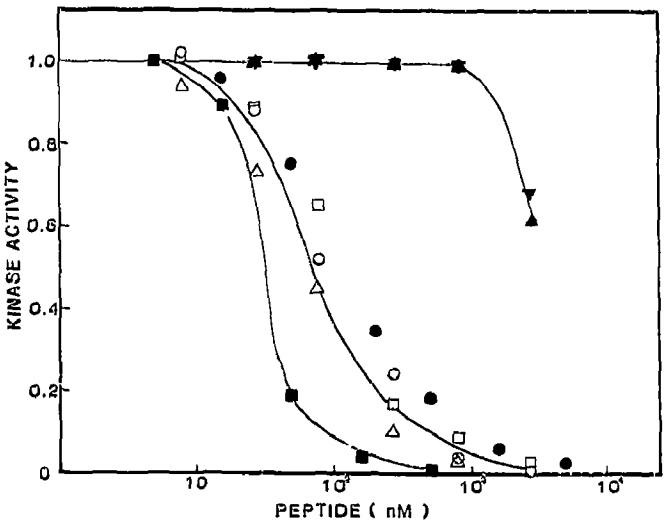

Fig. 1. Inhibition of constitutively active MLCK by the synthetic peptide analogs of autoinhibitory domain. 0.5 μg/ml of constitutively active MLCK was incubated with the synthetic peptides in the presence of 1 mM $MgCl_2$, 1 mM EGTA, 30 mM NaCl, 0.26 mg/ml smooth muscle S-1 and 30 mM Tris-HCl, pH 7.5, at 25°C for 5 min. The phosphorylation reaction was started by adding 0.2 mM [γ-^{32}P]ATP. The activity was estimated from the initial linear phase of the phosphorylation time course. The inhibition of the kinase activity in the presence of peptides was expressed by taking the activity in the absence of the peptides as 1.0. (○) peptide Ala-783–Gly-804; (△) peptide Ala-783–Lys-799; (□) peptide Ala-783–Lys-799 in which Lys-792 and Lys-793 are substituted for Ala; (●) peptide Lys-788–Lys-799; (■) peptide Ala-783–Arg-798; (▲) peptide Ala-783–Lys-799 in which Arg-797, Arg-798, and Lys-799 are substituted for Ala; (▼) peptide Ala-783–Lys-796.

Table I

Structure of the inhibitory peptides and their K_i against MLCK, IMLCK[a] and CaMPKII

Peptides		K_i (μM)[b]		
		IMLCK	MLCK[c]	CaMPKII[c]
783	804			
AKKLSKDRMKKYMARRKWQKTG		0.075	0.114	1.09
LSKDRMKKYMARRKWQK		0.060	0.110	1.05
AKKLSKDRMKKYMARRK		0.063	0.100	6.60
AKKLSKDRMKKYMARR		0.034	0.100	8.60
KDRMKKYMARR		0.115	0.302	4.37
AKKLSKDRMAAYMARRK		0.105	–	–
AKKLSKDRMKKYMA		3.000	–	–
AKKLSKDRMKKYMAAAA		3.600	–	–

[a] Constitutively active MLCK.
[b] K_i was estimated by measuring the kinase activity as a function of the inhibitory peptide concentration. Conditions are the same as Fig. 1.
[c] Assay was done in the presence of 0.1 μM calmodulin and 0.2 mM $CaCl_2$; otherwise the same as in Fig. 1. CaMPKII activity was measured using caldesmon as a substrate.

Because of the similarity between the autoinhibitory region and the myosin light chain phosphorylation site in terms of a cluster of basic residues, Kemp et al. [7] proposed that the autoinhibitory region acts as a pseudo-substrate inhibitor. Based on the substrate specificity studies using synthetic peptide analogs of the myosin light chain phosphorylation site, it was shown [17] that the basic residues in the light chain which correspond to Lys-788, Arg-790, Lys-792, Lys-793, Arg-797, Arg-798, Lys-799 and Lys-802 of the inhibitory region are important for substrate recognition and among them the last four residues are more important. A present study showed that Arg-797 and Arg-798 were important for the inhibitory activity but other basic residues were not essential for the inhibitory activity. This is distinct from the suggestion made by previous reports [7,18]. It has been shown that Arg-13 and Arg-16 of the regulatory light chain of smooth muscle myosin which correspond to Lys-799 and Lys-802 of MLCK are necessary for the substrate recognition of MLCK using synthetic peptide analogs [17] and truncated regulatory light chain [19]. This suggests that the inhibition of the kinase by the autoinhibitory region is not due to the simple pseudo-substrate mechanism.

Recently, it was reported [10,20] that the substitution of Arg-797–Lys-799 with glutamic acid residues by site-directed mutagenesis did not produce Ca^{2+}/calmodulin-independent kinase activity. This suggests that although Arg-797–Arg-798 are important for the inhibition of MLCK, the residues in the upstream of the autoinhibitory region also contribute to the inhibition of the kinase when the autoinhibitory region is incorporated in MLCK. Shoemaker et al. [10] reported that the substitution of the six basic residues of (Lys-783, -784, -788, -792, -793 and Arg-790) the upstream part of the autoinhibitory region with glutamic acid produced Ca^{2+}/calmodulin-independent activity. It was also suggested [21] that the cleavage at the N-terminal side of the cluster of the three basic residues, i.e. at the C-terminus of Arg-797 or Lys-792, produced an inactive form of MLCK which was converted to a constitutively active form by further proteolysis. This suggests that in the native protein, the N-terminal side of the autoinhibitory region may be important for the depression of enzyme activity although Arg-797 and Arg-798 may be also important for the inhibition of the activity.

Another interesting point for understanding the structure required for the regulation of MLCK is the structure required for calmodulin binding. We measured the attenuation of the inhibitory activity of the peptides by calmodulin to evaluate the calmodulin binding activity of the peptides (Fig. 2). Consistent with the earlier report [8], the inhibitory activity of the peptide Ala-783–Gly-804 was reversed by calmodulin. Deletion of Trp-800–Gly-804 decreased the calmodulin binding activity; however, significant calmodulin binding activity was still observed. Although the calmodulin binding

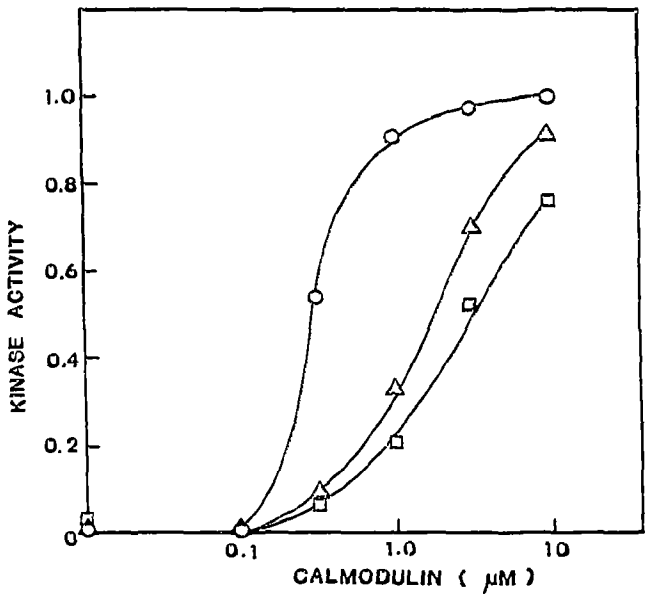

Fig. 2. Effect of calmodulin on the inhibition of constitutively active MLCK by the synthetic peptide analogs of autoinhibitory domain. Assay was done as described in Fig. 1, except 0.2 mM $CaCl_2$ and various concentrations of calmodulin were added instead of 1 mM EGTA. (○) 0.5 μM peptide Ala-783–Gly-804; (△) 0.5 μM peptide Ala-783–Lys-799; (□) 0.5 μM peptide Ala-783–Arg-798.

activity was a little decreased, deletion of an additional Lys-799 did not abolish the calmodulin binding activity. These results suggest that the calmodulin binding region and the autoinhibitory region overlap. Consistent with this result, the peptides also inhibited smooth muscle CaMPKII although a higher concentration was required for the inhibition (Table I). The inhibitory activity of these peptides against CaMPKII coincided with the calmodulin binding activity (Fig. 2); therefore, the inhibition is likely to be due to the calmodulin binding activity of the peptides. Actually the apparent K_i for the inhibition of CaMPKII by the peptides increased at higher calmodulin concentration (data not shown). Recently, Bagchi et al. [20] reported by analyzing the calmodulin binding to the mutant MLCK that the substitution of Lys-802, Arg-798–Lys-799 with alanine attenuates the calmodulin binding. The present results are consistent with their finding. The decrease in the affinity of the peptides for calmodulin by the deletion of the C-terminal side of the peptides is also consistent with the recent analysis of calmodulin–skeletal MLCK peptide complex by NMR which found that Trp-800 is important for the hydrophobic interaction between calmodulin and MLCK and the basic residues possibly contribute to the electrostatic interaction between calmodulin and MLCK [22].

Another significance of this study is to develop the specific peptide inhibitor of MLCK. We previously reported [11] that the peptide inhibitor of MLCK (residues 783–804) inhibits the contraction of the isolated

single smooth muscle cells after the high K^+-induced stimulation although the transient increase in the cytoplasmic Ca^{2+} after the stimulation is rather increased by the peptide injection. As shown in Table I, although higher concentration was required the peptide Ala-783–Gly-804 also inhibited CaMPKII. Since MLCK is thought to be a quite substrate-specific kinase and known substrate is only the regulatory light chain of myosin while CaMPKII is known to have a broad substrate specificity, it is likely that the observed enhancement of the transient increase in the cytoplasmic Ca^{2+} is likely to be due to the inhibition of CaMPKII by the peptide. The truncation of the C-terminal side of the peptide decreased the calmodulin binding activity (Fig. 2), therefore, it is expected that the shorter peptides such as Ala-783–Arg-798 serve as a more specific inhibitor.

Acknowledgements: This work was supported by Public Health Service Grant AR38888 and Grant-in-Aid from American Heart Association and with funds contributed in part by the AHA Northeast Ohio Affiliate.

REFERENCES

[1] Hartshorne, D.J. (1987) in: Physiology of the Gastrointestinal Tract (Johnson, L.R. ed.) 2nd edn., pp. 432–482, Raven Press, New York.
[2] Sellers, J.R. and Adelstein, R.S. (1987) in: The Enzymes (Boyer, P. and Krebs, E.G. eds.) vol. 18, pp. 381–418, Academic Press, San Diego, CA.
[3] Guerriero Jr., V., Russo, M.A., Olson, N.J., Putkey, J.A. and Means, A.R. (1986) Biochemistry 25, 8372–8381.
[4] Olson, N.J., Pearson, R.B., Needleman, D.S., Hurwitz, M.Y., Kemp, B.E. and Means, A.R. (1990) Proc. Natl. Acad. Sci. USA 87, 2284–2288.
[5] Gallagher, P.J., Herring, P.B., Griffin, S.A. and Stull, J.T. (1991) J. Biol. Chem. 266, 23936–23944.
[6] Lukas, T.J., Burgess, W.H., Prendergast, F.G., Lau, W. and Watterson, D.M. (1986) Biochemistry 25, 1458–1464.
[7] Kemp, B.E., Pearson, R.B., Guerriero Jr., V., Bagchi, I.C. and Means, A.R. (1987) J. Biol. Chem. 262, 2542–2548.
[8] Ikebe, M., Stepinska, M., Kemp, B.E., Means, A.R. and Hartshorne, D.J. (1987) J. Biol. Chem. 262, 13828–13834.
[9] Herring, B.P. (1991) J. Biol. Chem. 266, 11838–11841.
[10] Shoemaker, M.O., Lau, W., Shattuck, R.L., Kwiatkowski, A.P., Matrisian, P.E., Guerra-Santos, L., Wilson, E., Lukas, T.J., Van Eldik, L.J. and Watterson, D.M. (1990) J. Cell Biol. 111, 1107–1125.
[11] Ito, T., Ikebe, M., Kargacin, G., Hartshorne, D.J., Kemp, B.E. and Fay, F.S. (1989) Nature 338, 164–167.
[12] Walsh, M.P., Hinkins, S., Dabrowska, R. and Hartshorne, D.J. (1983) Methods Enzymol. 99, 279–288.
[13] Ikebe, M., Reardon, S., Scott-Woo, G.C., Zhou, Z. and Koda, Y. (1990) Biochemistry 29, 11242–11248.
[14] Ikebe, M. and Hartshorne, D.J. (1985) J. Biol. Chem. 260, 13146–13153.
[15] Ikebe, M. and Hartshorne, D.J. (1985) Biochemistry 24, 2380–2387.
[16] Bretscher, A. (1984) J. Biol. Chem. 259, 13873–13880.
[17] Kemp, B.E., Pearson, R.B. and House, C.M. (1983) Proc. Natl. Acad. Sci. USA 80, 7471–7475.
[18] Pearson, R.B., Ito, M., Morrice, N.A., Smith, A.J., Condron, R., Wettenhall, R.E.H., Kemp, B.E. and Hartshorne, D.J. (1991) Eur. J. Biochem. 200, 723–730.
[19] Ikebe, M. and Morita, J.-I. (1991) J. Biol. Chem. 266, 21339–21342.
[20] Bagchi, I.C., Huang, Q. and Means, A.R. (1992) J. Biol. Chem. 267, 3024–3029.
[21] Ikebe, M., Maruta, S. and Reardon, S. (1989) J. Biol. Chem. 264, 6967–6971.
[22] Ikura, M., Clore, G.M., Gronenborn, A.M., Zhu, G., Klee, C.B. and Bax, Z. (1992) Science 256, 632–638.